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(54) Title: STEM CELL THERAPY

(57) Abstract: The present invention provides a method of culturing a teratoma-like cell mass in conditions which are suitable for the further growth and/or differentiation of teratoma derived cells. The invention further relates to a method to produce differentiated and/or committed cells and/or tissues, their therapeutic use in tissue engineering and cell/tissue transplantation; cellular compositions comprising the differentiated cells and methods for therapeutic interventions with the cells.

STEM CELL THERAPY

FIELD OF THE INVENTION

This invention relates to a method to produce differentiated and/or committed cells and/or tissues, their therapeutic use in tissue engineering and cell/tissue transplantation; cellular compositions comprising said differentiated cells and methods for therapeutic interventions with said cells.

BACKGROUND OF THE INVENTION

Animal embryonic development combines cell proliferation and cell/tissue differentiation to produce an intact organism and is a highly regulated developmental process. Mammalian embryonic development is remarkably conserved during the early stages. Post fertilisation the early embryo completes four rounds of cleavage to form a morula of 16 cells. After several more rounds of division these cells then develop into a blastocyst. Cells in a blastocyst can be divided into two distinct regions: the inner cell mass, which will form the embryo, and the trophectoderm, which will form extra-embryonic tissue such as the placenta.

Cells that form part of the embryo up until the formation of the blastocyst are totipotent. This means that each cell has the developmental potential to form a complete embryo and all the cells required to support the growth and development of that embryo. In contradistinction, a pluripotent or multipotent cell has the developmental potential to form a variety of tissues. During blastocyst formation, the cells that comprise the inner cell mass are said to be pluripotential.

A blastocyst undergoes further development to give rise to a blastula which comprises the three primary germ layers (ectoderm, endoderm and mesoderm). It is from these germ layers that the principal cell types of the body are derived, e.g., nervous, epithelial, connective and muscular cells. It is known which germ layer each body cell type is derived from.

These principal cell types represent the primary trends of differentiation from the earliest generalised cells of the embryo and their appearance, which differs more and more as they multiply to form the first embryonic tissues, is marked by a selective reduction in the potentialities coded in the DNA content of their nuclei. The different levels of cells are however not absolutely fixed and under certain conditions cells may alter their characteristics surprisingly. Each of the cell categories constitutes a group in which many variations of the fundamental cell type occurs but with an underlying similarity of behaviour.

Pluripotent embryonic cells can be isolated from two embryonic sources. Cells derived from the inner cell mass of the blastocyst formed during early embryogenesis are termed embryonic stem cells (ES cells). Embryonic germ cells (EG cells) are pluripotent cells collected from fetal tissue at a somewhat later stage of development, i.e., primordial germ cells isolated from the mesenteries or genital ridges of days 8.5-12.5 *post coitum* embryos which would ultimately differentiate into germ cells. As used herein the term pluripotential cell refers equally to ES and/or EG cells.

Each of these types of pluripotent cell has the same developmental potential with respect to differentiation into alternate cell types. Importantly, pluripotential cells have an increased commitment to terminal differentiation when compared to a totipotent cell. An intact embryo cannot be produced from a single ES or EG pluripotential cell.

Adult stem cells obtained from mature tissues differentiate into a narrower range of cell types. Accordingly many cells of medical interest might not be obtained from adult-born stem cells. It is also less feasible to develop large-scale cultures from adult stem cells.

A potentially limitless source of cells, stem cells can both duplicate themselves and produce differentiated cell types that constitute the various tissues or organ systems of the human body. The use of *in vitro* cultures of pluripotential stem cells and their

tissue-committed derivatives, particularly human cells, has important ramifications in transplantation and/or replacement therapies (regenerative medicine) for critical tissues which have been damaged through injury or disease. Because of their pluripotent nature, ES and EG cells can differentiate into a variety of cell types and/or tissues and organs. Potential applications include, by no way of limitation, treatment of various severe pathological conditions such as neurodegenerative diseases (neuronal reconstitution in Parkinson's, Alzheimer, MS and ALS), neurological and neurosensorial pathological conditions (spinal cord injury, cerebral ischemia/stroke, retina diseases), endocrine metabolic diseases (islet body reconstitution in diabetes, pituitary diseases, hepatic failure) and cardiovascular diseases (myocardial tissue reconstitution post-MI, cardiac heart failure and coronary heart diseases).

In order to establish a pluripotent cell-line which, upon exposure to various differentiation factors, can lead to the production of selected differentiated tissue for use, *inter alia*, in transplantation therapy, *in vitro* cultures of human ES/EG cells must be established. This has proved to be particularly problematical.

US 5 453 357 and US 5 690 926 describe the establishment of *in vitro* cultures of ES/EG cells which are derived from non-murine species. Typically the ES/EG cultures have well defined characteristics. These include, but are not limited to;

(i) maintenance in culture for at least 20 passages when maintained on fibroblast feeder layers;

(ii) production of clusters of cells or 'embryoid bodies' in culture;

(iii) ability to differentiate into multiple cell types in monolayer culture;

(iv) formation of embryo chimeras when mixed with an embryo host;

(v) expression of ES/EG cell specific markers.

WO 96/22362 discloses cell-lines and growth conditions which allow the continuous proliferation of primate ES cells and provides the first indication that conditions which allow the establishment of human ES/EG cells in culture may be determined.

The ES cells disclosed in WO 96/22362 exhibit a range of characteristics or markers associated with their pluripotential characteristics such as the expression of specific cell markers SSEA-1 (1), SSEA-3 (+), SSEA-4 (+), TRA-1-60 (+), and TRA-1-81 (+) (Shevinsky et al 1982; Kannagi et al 1983; Andrews et al 1984), alkaline phosphatase (+) and combinations thereof. The established primate cell-lines have stable karyotypes and continue to proliferate in an undifferentiated state in continuous culture. Importantly the primate ES cell-lines also retain the ability, throughout their continuous culture, to form tissues derived from all three embryonic germ layers (endoderm, mesoderm and ectoderm).

Pluripotential cells are also characterised by a characteristic chromosomal methylation pattern. The eukaryotic genome is variably methylated through the addition of methyl ($-CH_3$) groups which are attached to cytosine residues in DNA to form 5'methylcytosine (5'-mC). Methylation is correlated with the control of gene expression whereby genes that are hypomethylated tend to be highly expressed. Hypermethylation is correlated with reduced gene expression. Pluripotential cells will have a typical methylation pattern which may be analysed at a genomic level or at the level of a specific gene.

Thomson *et al* 1998 disclose conditions in which human ES cells can be established in culture. The human cell-lines share the above characteristics which are shown by primate ES cells and show high levels of telomerase activity which confers the ability of continuous division in culture. Telomerase enzymes add, *de novo*, repetitive DNA sequences to the ends of chromosomes. These ends are referred to as telomeres. The telomeres of human chromosomes contain the sequence '5 TTAGGG 3' repeated approximately 1000 times at their ends. In young, dividing cells the telomeres are relatively long. In ageing, or non dividing cells, the telomeres become shortened and there is a strong correlation between telomere shortening and proliferative capacity. Methods to increase telomere length and proliferative capacity are described in WO9513383. Telomerase enzymes and telomerase activity are constitutively highly expressed in haematopoietic stem cells of cord origin.

The establishment of human EG cell cultures is disclosed in WO 98/43679. EG cells were isolated from the gonadal or genital ridges of human embryos and were found to exhibit continuous proliferation in culture in an undifferentiated state, normal karyotype and the ability to differentiate into selected tissues under defined conditions.

It is well known that amphibian somatic cell nuclei retain their ability to give rise to entire embryos when transplanted into enucleated oocytes (Gurdon 1974). Determination of the pluripotency of these cells must be controlled by the egg cytoplasm which can 'reprogram' the somatic cell nucleus into a totipotent state. This effect has also been observed in the transfer of mammalian somatic cell nuclei to enucleated oocytes wherein the nuclei retain this plasticity and can be reprogrammed, (Campbell *et al.*, Wakayama *et al*). The material produced is genetically identical to the somatic cell donor.

GB 2318578 discloses the use of nuclei from differentiated or partially differentiated cells in nuclear transfer wherein gene expression of the transferred nuclei is reprogrammed to 'bring out' their inherent totipotency. In contrast to the present invention, GB 2318578 is concerned with embryo reconstitution and production of whole organisms therefrom. The document highlights the difficulties in promoting development to term following the use of ES cells in nuclear transfer. The present invention relates specifically to the use of nuclei from haematopoietic stem cells due to their inherent biological nature (high plasticity, high telomerase activity and DNA methylation which, as a whole, confer high proliferative capacities), as source for tissue-committed stem cells.

Other studies have emphasised the role of cell cycle co-ordination of the donor nucleus and recipient cytoplasm in the development of embryos reconstructed by nuclear transfer (Campbell *et al.*, Biol. Reprod. **49** 933-942 (1993) and Biol. Reprod. **50** 1385-1393 (1994)).

The mitotic cell cycle comprises four distinct phases; G1, S, G2 and M. Initiation of the cell cycle (start) occurs in the G1 phase and it is here that the decision to undergo another cell cycle is made. The remainder of the G1 phase is the pre-DNA synthesis phase. DNA synthesis takes place in the S phase and is followed by the G2 phase, which is the period between DNA synthesis and mitosis respectively. Mitosis occurs at M phase followed by cytokinesis. Quiescent cells, however, are those which are not actively dividing and are described as being in the G0 state.

A number of metabolic changes have been reported in quiescent cells. These include monophosphorylated histones, ciliated centrioles, reduction or complete cessation in all protein synthesis, increased proteolysis, decrease in transcription and increased turnover of RNA resulting in a reduction in total cell RNA, disaggregation of polyribosomes, accumulation of inactive 80S ribosomes and chromatin condensation (reviewed Whitfield et al, *Control of Animal Cell Proliferation*, 1 331-365 (1985).

GB 2318578 discloses the use of quiescent cells, i.e., those which are not actively proliferating by means of the mitotic cell cycle, as nuclear donors in the reconstitution of an animal embryo. Changes that occur in the donor nucleus which are observed after embryo reconstruction and which are required for efficient nuclear transfer are induced in the nuclei of cells prior to their use as nuclear donors by causing them to enter the quiescent state. Quiescent cells have a very limited proliferative potential due to their inherent chronological status (absence of telomerase activity).

The cells which form tumours referred to as teratomas have many features in common with ES cells. The most important of these features is the characteristic of pluripotentiality.

Teratomas contain a wide range of differentiated cells and tissues, and have been known in humans for many hundreds of years. They typically and clinically occur as gonadal or extra-gonadal tumours in both men and women. The gonadal forms of

these tumours are generally believed to originate from germ cells, and the extra gonadal forms, which typically have the same range of tissues, are widely thought to arise from germ cells that have migrated incorrectly during embryogenesis. Teratomas are therefore generally classed as germ cell tumours which encompass a number of different types of dysplastic and/or neoplastic cells.

Ovarian germ cell tumours are most commonly benign and contain only well differentiated somatic cells and tissues that may include bone, muscle and nerve. It is often the case that well organised tissues are found including teeth and hair. By contrast, human testicular germ cell tumours are often neoplastic. Testicular germ cell tumours also produce differentiated cells and tissues, although these may be less well organised than compared to the benign ovarian germ cell tumours.

The transplantation of blastocyst to ectopic sites results in the formation of teratomas. Naturally occurring teratomas are of limited value. They exhibit a number of undesirable characteristics. The genome of a teratoma cell includes mutation(s) in gene(s) which result in the transformed phenotype. The karyotype of the teratoma cell is unstable resulting in a euploid or polyploid chromosome complement. However, as noted above, teratoma cells retain pluripotential characteristics. Typically, these tumours are detected after many years from their biological constitution.

We propose the generation of "teratoma-like" cell masses by the in-vitro nuclear transplantation of a somatic nucleus (from either a non-quiescent or quiescent somatic cell) into an enucleate oocyte followed by in-vitro oocyte activation and development into a morula-like structure, and the subsequent ectopic (i.e. subcutaneous) transplantation of the said cell mass into a recipient individual (human or animal), ideally the same subject to whom the nucleus belongs. This methodology will result in the provision of differentiated cells or tissues for further in vitro cell/tissue engineering processes for therapeutic purposes. The cell mass will be allowed to develop in vivo into a localised microscopic teratoma before its excision

and further processing and growth in cell/tissue culture systems. The use of somatic nuclei (either non-quiescent or quiescent), derived from cells of the individual who will subsequently receive the differentiated cells or tissues, is advantageous since the majority of the cells will be almost entirely autologous, although the enucleated oocyte will maintain its own phenotype. However, the latter should not compromise its immune privileged status. Moreover, the above identified disadvantages associated with naturally occurring teratomas will be avoided because the nuclei used to derive the developing cell mass will not carry mutations and will have a stable genotype.

The teratoma-like cells can be established in tissue culture or as cell-lines depending on the particular cell/tissue type. An individual can establish and maintain a cell/tissue bank (e.g. cryopreserved haematopoietic stem cells of cord origin or during adulthood) which can be drawn upon as and when the individual requires replacement of critically diseased and/or damaged tissue.

Some examples of the therapeutic applications and value of this approach are the establishment, through the above technology, of autologous dopaminergic neurons or pre-neurons to be used for neurosurgical transplantation into the degenerated substantia nigra of patients with Parkinson's disease; the transplantation of autologous neurons in other neurodegenerative disorders (Multiple Sclerosis, ALS); the transplantation of autologous pre-neurons or neurons in patients with spinal cord injury; the replacement of CNS neurons lost following stroke. Another area of critical intervention is the establishment of autologous Langerhans beta-cells producing insulin or entire islet of Langerhans producing counteracting hormones such as glucagon, and their implantation in patients with diabetes. Or the establishment of striated myocardial cells to be injected, through coronary catheterisation, into an acute area of myocardial infarction.

STATEMENTS OF THE INVENTION

According to the present invention there is provided method of culturing cells derived by growing a cell mass to form a teratoma-like cell mass, to grow and/or differentiate said cells; for example this may be a method of culturing a teratoma-like cell mass in *in-vitro* conditions which are suitable for the further growth and/or differentiation of teratoma derived cells.

Preferably the invention comprises providing an activated oocyte obtained by nuclear transfer from a somatic donor cell, either quiescent or non-quiescent (e.g. haematopoietic stem cell from peripheral blood, bone marrow or from cryopreserved cord blood stem cells) to a recipient oocyte and, before or after transfer, removing the oocyte nucleus, providing conditions which are suitable for the division of the oocyte to at least a sixteen-cell mass (morula-like) stage, transplanting the cells into a recipient (i.e. subcutaneously) and allowing the cells to develop into a localised teratoma-like cell massPreferably the method further comprises excising the teratoma-like cell mass.

According to an alternative embodiment of the invention, there is provided a method to produce a pluripotent cell, the method comprising transferring a hematopoietic donor stem cell nucleus into a recipient oocyte and, before or after transfer, removing the oocyte nucleus.

DETAILED DESCRIPTION OF THE INVENTION

Embodiments of the invention will now be described by example only and with reference to the following materials and methods.

1. Teratoma-like cell mass

This embodiment of the invention preferably comprises the following steps:

(i) transferring the nucleus of a somatic donor cell (which may be either quiescent or non-quiescent, e.g. a haematopoietic stem cell from peripheral blood, bone marrow or from cryopreserved cord blood stem cells) into a recipient enucleated oocyte;

(ii) activating the oocyte;

(iii) providing conditions which are suitable for the division of the oocyte to at least a sixteen-cell mass (morula-like stage);

(iv) providing conditions which are suitable the sixteen-cell mass to develop into a localised teratoma-like cell mass;

(v) separating out differentiated cells of the cell mass, optionally either before or after culturing the cells in in-vitro conditions which are suitable for the further growth and/or differentiation thereof.

Step (v) typically comprises transferring the sixteen-cell mass to a recipient where the cells are allowed to develop into a localised teratoma-like cell mass. The recipient may be an animal, for example an immunosuppressed pig, or less preferably an *ex vivo* growth environment.

The invention also includes a method comprising steps (i) to (iv) above, and optionally also step (v). Further included in the invention is performance of step (v) on cells derived from the cell mass in (iv).

The invention therefore further provides a method comprising:

(i) obtaining a somatic cell, either quiescent or non-quiescent (e.g. haematopoietic stem cell from peripheral blood, bone marrow or from cryopreserved cord blood stem cells)

(ii) transferring the nucleus of the somatic cell into a recipient enucleated oocyte;

(iii) activating the oocyte;

(iv) providing conditions which are suitable for the division of the oocyte to at least a sixteen-cell mass (morula-like stage);

(v) transplanting the cells into a recipient (i) (i.e. subcutaneously);

(vi) allowing the cells in (v) to develop into a localised teratoma-like cell mass;

(vii) excising the teratoma – like cell mass in (vi); and

(viii) providing in-vitro conditions which are suitable for the further growth and/or differentiation of teratoma derived cells.

The invention also includes a method comprising steps (i) to (iv) above, and optionally also step (v), optionally followed by step (vi), optionally followed by step (vii). Further included in the invention is performance of step (viii) on cells derived from the cell mass in (vii).

In practice, step (i) will often be performed separately from the remaining steps, to form a cell/tissue bank from which donor cells can be drawn, so that the process in these cases can be said to start with step (ii).

In a preferred method of the invention, said donating cell is autologous.

In an alternative method of the invention, said donating cell is allogenic.

The terms autologous and allogenic are well known in the art. For the sake of clarity, autologous refers to a cell obtained from an individual, the nucleus of which is to be

used for nuclear transplantation and subsequent cell mass and teratoma development. Allogenic refers to a cell obtained from another individual, the nucleus of which is to be used in the above procedure.

In a further preferred method of the invention, said donating cell is quiescent. Preferably said quiescent cell is selected from any type of quiescent somatic cell (e.g. fibroblast).

In an alternative preferred embodiment of the invention, said donating cell is non-quiescent. Preferably, said non-quiescent cell is selected from haematopoietic stem cells and/or lymphopoietic stem cells. The use of a haematopoietic stem cell ('HSC') as donor is itself an invention and is described further below under the heading 'HSC as donor'.

It will be apparent that haemopoietic stem cells (HSC with a CD34+, Lin – phenotype) can be isolated from either autologous cord blood or bone marrow/peripheral adult blood (see below under the heading 'HSC as donor' for more information). Non quiescent HSC's have advantages over quiescent somatic cells. For example they maintain an undifferentiated state, express high levels of telomerase, DNA remains in an unmethylated state maintaining the proliferative and developmental potential. Furthermore the autologous teratoma derived tissues when implanted will not be subjected to immune rejection therefore removing the need to administer immunosuppressive drugs.

Methods to grow haematopoietic stem cells are known in the art. For example, US5728581 describes bioreactors for expanding haematopoietic stem cells which involves the provision of culture conditions which promote the expansion of stem cells by combining variations in oxygen tension and addition of at least one cytokine which stimulates proliferation.

Collection of neonatal blood and selection of HSC

Hematopoietic cells (essentially CD34+ Lin-) are isolated from neonatal blood contained in the cord/placenta. The cells can be obtained easily and without trauma to the donor. EP0343217 describes methods to obtain hematopoietic stem cells from the umbilical cord blood by immediate cord clamping after delivery and direct drainage. Volumes of 50 ml or more of neonatal blood were obtained and found to contain enough of the appropriate cells to repopulate the entire hematopoietic system of an adult with an appropriate weight.

The method of the present invention provides for autologous (self) reconstitution and allogeneic cell/tissue transplantation whereby the nucleus of the hematopoietic stem cell is derived from another individual. Although autologous (self) reconstitution eliminates all risk of immune rejection, it will be apparent that this route will be most useful in the repair of injury, trauma or critical degeneration such as damage to the spinal cord, PD, CNS degenerative conditions, MI, diabetes, bone reconstitution. The allogeneic route may be preferred in circumstances where an individual has a genetic predisposition to disease or known genetic conditions impeding the autologous route.

Cryopreservation

Procedures and considerations for the manipulation, cryopreservation and long term storage of hematopoietic stem cells are well known in the art. Whole neonatal blood, as collected, is cryogenically frozen using known techniques.

Standard cell separation procedures and *in-vitro* stem and progenitor cell expansion are preferably carried out before cryopreservation to reduce sample volume and increase cell count respectively. In one embodiment of the invention, HSC cells are separated from other blood components and cryopreserved. In an alternative embodiment, the hematopoietic stem cells may be enucleated before cryopreservation

and only the nuclei (nucleoplasts or karioplasts) cryopreserved under suitable conditions. Cryopreserving nucleoplasts instead of the entire cell, will cause a lower "cryogenic shock" due to their inherent lower water content (virtually no formation of water microcrystals) and lower concentration of cryopreservants. Furthermore, the absence of mitochondrial structures, which are abundant in the whole cell, will result in a lower oxidative stress which is a negative effect during the thawing out. In another embodiment of the invention, a reconstituted oocyte containing a HSC stem cell nucleus is cryopreserved.

EP 0343217 describes methods for recovering stem and progenitor cells from the frozen state. Accordingly, cryopreservation of the HSC nuclei provides a stem cell bank which can be tapped when and if needed to provide a HSC nucleus for use in nuclear transfer into an enucleated oocyte. In an alternative embodiment of the invention, the HSC nucleus is transferred into an intact oocyte, following which the oocyte nucleus is removed.

Enucleation of cells to yield 'karyoplasts' and 'cytoplasts'

Selected treatment of cells in culture can result in cells extruding nuclei, resulting in the formation of separate nuclear and cytoplasmic parts named karyoplasts and cytoplasts respectively. In a preferred embodiment of the present invention, the recipient is enucleate. While it has been generally assumed that enucleation of recipient oocytes in nuclear transfer procedures is essential, there is no published experimental confirmation of this judgement. Enucleation may be achieved physically, by removal of the nucleus, pro-nuclei or metaphase plate (depending on the recipient cell). It may also be achieved functionally, such as by the application of ultraviolet radiation or another enucleating influence.

Different procedures have been used in attempts to remove the chromosomes with a minimum of cytoplasm. Aspiration of the first polar body and neighbouring cytoplasm was found to remove the metaphase II apparatus in 67% of sheep oocytes (Smith & Wilmut *Biol. Reprod.* 40 1027-1035 (1989)). Only with the use of DNA-

specific fluorochrome (Hoechst 33342) was a method provided by which enucleation would be guaranteed with the minimum reduction in cytoplasmic volume (Tsunoda et al., *J. Reprod. Fertil.* 82 173 (1988)). In livestock species, this is probably the method of routine use at present (Prather & First *J. Reprod. Fertil. Suppl.* 41 125 (1990), Westhusin et al., *Biol. Reprod. (Suppl.)* 42 176 (1990)).

There have been very few reports of non-invasive approaches to enucleation in mammals, whereas in amphibians, irradiation with ultraviolet light is used as a routine procedure (Gurdon *Q. J. Microsc. Soc.* 101 299-311 (1960)). There are no detailed reports of the use of this approach in mammals. During the use of DNA-specific fluorochrome however, it was noted that exposure of mouse oocytes to ultraviolet light for more than 30 seconds reduced the developmental potential of the cell (Tsunoda et al., *J Reprod. Fertil.* 82 173 (1988)).

Enucleation results in a cell lacking a nucleus, but is otherwise intact for a number of days (Goldman et al 1973) These enucleated cells have been called anucleate cells (Poste 1972) or cytoplasts (Veomett et al 1974). The nucleus that is extruded from the cell retains a thin rim of cytoplasm and is surrounded by a plasma membrane; these structures have been called 'karyoplasts' (Veomett et al 1974) or 'mini-cells' (Ege and Ringertz 1975).

The nucleus of the hematopoietic stem cell may be separated by exposure to a pharmacologically effective amount of cytochalasin B. Cytochalasin B is an example of a chemical which is effective at separating the nucleus of a cell from the cytoplasm to form a karyoplast and cytoplast respectively, (Methods in Enzymology Vol 151, p221-237 1987). In a well-established technique, cells attached to a plastic disc are inverted over a solution of cytochalasin B in a centrifuge tube and centrifuged. The cytoplasts remain attached to the plastic disc, while the karyoplasts are pelleted at the bottom of the centrifuge tube (Prescott et al 1972).

Alternatively, cells in suspension may be centrifuged through a density gradient, typically composed of Ficoll, containing cytochalasin B (Wigler and Weinstein

1975). In this case, cytoplasts and karyoplasts are formed and may be recovered from different parts of the gradient after centrifugation.

Any of the above techniques may be used to generate a donor nucleus which is preferably derived from a hematopoietic stem cell and a recipient oocyte cytoplast.

In one embodiment of the invention, the donor cell and recipient oocyte are of human origin. In an alternative embodiment, the donor cell and recipient oocyte are of non-human mammalian origin.

Transfer of the donor nucleus to an oocyte

The recipient cell to which the nucleus from the donor cell is transferred is an oocyte. In a preferred embodiment of the invention, a HSC nuclei is transferred into an enucleated oocyte obtained by any of the above described techniques. In an alternative embodiment of the invention, the HSC nucleus is transferred into an intact oocyte. The oocyte nucleus is then removed accordingly. The oocyte is of human or mammalian origin.

It is preferred that the recipient host cell to which the donor cell nucleus is transferred is an enucleated metaphase II oocyte, an enucleated unactivated oocyte or an enucleated preactivated oocyte. At least where the recipient is an enucleated metaphase II oocyte, activation may take place at the time of transfer. Alternatively, at least where the recipient is an enucleated unactivated metaphase II oocyte, activation may take place subsequently.

Three suitable cytoplast (enucleated oocyte) recipients are:

1. The "MAGIC Recipient" (Metaphase Arrested G1/G0 AcceptIng Cytoplast) described in WO9707668.

2. The "GOAT" (GO/G1 Activation and Transfer) – a MII (metaphase II) oocyte at the time of activation (Cambell et al., *Biol. Reprod.* 49 933-942 (1993).

3. The "Universal Recipient" (Campbell et al., *Biol. Reprod.* 649 933-942 (1993), *Biol. Reprod.* 50 1385-1393 (1994).

All the above publications are included herein by reference.

Once suitable donor and oocyte cells have been identified, it is necessary for the nucleus of the former to be transferred to the latter. Nuclear transfer is effected by fusion or micromanipulation.

Three established methods which have been used to induce fusion are:

(1) exposure of cells to fusion-promoting chemicals, such as polyethylene glycol;

(2) the use of inactivated virus, such as Sendai virus; and

(3) the use of electrical stimulation.

Methods for creating hybrid cells by fusing two or more cells of different origins together have been well documented. Kennett 1979 provides a review of the commonly used methods based upon Sendai virus induced cell fusion, or cell fusion induced by polyethylene glycol (PEG).

Cells to be fused are incubated with a fusogenic agent, such as Sendai virus or PEG. Centrifugation or agitation may be used to encourage clumping and close apposition of the cell membranes. Variables such as time, temperature, cell concentration and fusogenic agent concentration are optimised for each cell combination. These techniques have been shown to allow fusion of cytoplasts prepared by cytochalasin B induced enucleation and with whole cells or karyoplasts, also derived by cytochalasin

B induced enucleation (Poste and Reeve 1971; Ege and Ringertz 1975; Ege et al 1973, 1974; Veomett et al 1974; Wright and Hayflick 1975; Shay 1977)).

Electrofusion is another well established and widely used method for inducing cell fusion. This involves passing short electric pulses through mixtures of cells (Neil and Zimmermann 1993).

While cell-cell fusion is a preferred method of effecting nuclear transfer, it is not the only method that can be used. Other suitable techniques include microinjection (Ritchie and Campbell, *J Reproduction and Fertility* Abstract Series No. 15, p60).

During fertilisation there are repeated, transient increases in intracellular calcium concentration (Cutbertson & Cobbold *Nature* 316 541-542 (19185). Electrical pulses are believed to cause analogous increases in calcium concentration. There is evidence that the pattern of calcium transients varies with species and it is anticipated that the optimal pattern of electrical pulses will vary in a similar manner.

Exposure of rabbit oocytes to repeated electrical pulses reveals that selection of an appropriate series of pulses and control of Ca^{2+} is necessary to promote development of diploidized oocytes to mid-gestation (Ozil *Development* 109 117-127 (1990)). The interval between pulses in the rabbit is approximately 4 minutes (Ozil *Development* 109 117-127 (1990)), and in the mouse 10 to 20 minutes (Cutbertson & Cobbold *Nature* 316 541-542 (1985)). There are preliminary observations in the cow to suggest that the interval is approximately 20 to 30 minutes (Robl et al., in *Symposium on cloning Mammals by Nuclear Transplantation* (Seidel ed.), Colorado State University, 24-27 (1992)). In most published experiments activation was induced with a single electrical pulse. New observations suggest that the proportion of reconstituted embryos that develop is increased by exposure to several pulses (Collas & Robl *Biol. Reprod.* 43 877-884 (1990)). In any individual case, routine adjustments may be made to optimise the number of pulses, field strength, pulse duration and calcium concentration of the medium.

Cell division

The cell is simply allowed to develop without further intervention beyond any that may be necessary to allow the development of a blastocyst-type mass to take place.

Alternatively or additionally, the cell mass may be split and the cells clonally expanded to improve yield. Accordingly, the method of the present invention provides means for the preparation of a new cell-line which may act as a source of nuclear donor cells which can be produced from a cell mass formed according to the preceding description. Increased yields of cells may alternatively or additionally be obtained by clonal expansion of donors and/or by use of the process of serial (nuclear) transfer.

The cell mass derived by nuclear transfer is not an embryo (special culture conditions would normally be required *in vivo*) but *in vitro* conditions routinely employed in the art are quite acceptable. The developing cell mass undergoes development to give rise to a blastula-type mass which comprises the three primary germ layers (ectoderm, endoderm and mesoderm) from which a cell or cell-line of the present invention is derived.

Teratoma-like cell mass development

The blastula-type mass is allowed to grow into a teratoma-like mass in conditions suitable for such growth. Usually, but not necessarily, the blastula-type mass is transplanted into a recipient human or non-human animal. Optionally, this step includes the encapsulation of the cell-mass in a biocompatible carrier. It will be apparent to one skilled in the art that the biocompatible carrier serves to retain the teratoma-like cells to prevent migration from the site of transplantation to prevent the spreading of cells. However the carrier will be such that the diffusion of nutrients/ growth factors to the growing teratoma is not impeded. The fact that only a limited period of time is allowed in-vivo, further avoids the risk of neoplastic transformation.

Biocompatible carriers are known in the art. For example, US5976780 describes an encapsulation device for cells for use in tissue transplantation or tissue implantation. The device is manufactured from porous sodium alginate and polysulfone fibres which facilitate the diffusion of nutrients required to maintain cells is a functional state.

The recipient may be an immunosuppressed selected animal model (syngenic, transgenic or cloned/transgenic animal suitable for xenotransplantation).

As another alternative, the blastula-like mass can be transferred to an ex-vivo biological surrogate in which the implantation into the recipient is substituted with a segregated tissue compartment from the same recipient (e.g. skin strip or muscle strip with appropriate nutrients/growth factors), which allows the development of the morula-like mass into more differentiated and committed cells.

The cell mass is allowed to develop (either *in* or *ex vivo*) into a localised microscopic teratoma.

Excision

In preferred methods the teratoma-like cell mass is allowed to grow in a recipient animal or *ex vivo* tissue (e.g. segregated human tissue compartment) and excised from the recipient or tissue.

Further processing and growth in cell/tissue systems

Separation of differentiated cells is achieved by standard techniques and, if desired, may be carried out either before or after allowing further growth and/or differentiation of the teratoma cells.

The invention additionally provides a cell or cell-line derived from a teratoma-like cell mass formed by the method according to the invention.

In a preferred embodiment of the invention, said cell is a differentiated cell selected from: neural cell; muscle cell (e.g. smooth, striated, cardiac); bone (osteoblast, osteoclast); chondrocyte; hepatocyte; renal; respiratory epithelium; haematopoietic cells; spleen cell; pancreatic islets (α, β cells); endothelial cells.

According to a further aspect of the invention there is provided an organ or tissue comprising a cell according to the invention.

According to yet a further aspect of the invention there is provided a method to obtain therapeutic polypeptides expressed by the cells according to the invention comprising:

i) providing cell culture conditions suitable for the growth of a cell according to the invention;

ii) isolating from said cell or the culture environment a therapeutic polypeptide; and, optionally;

iii) purifying and storing said therapeutic polypeptide.

According to a further aspect of the invention there is provided a therapeutic composition comprising at least one therapeutic polypeptide according to the invention.

According to a further aspect of the invention, there is provided a therapeutic composition comprising a cell according to the invention. Preferably, said cell is for use in the manufacture of replacement tissue for use in tissue engineering.

According to a yet further aspect of the invention there is provided a cell according to the invention encapsulated in a biocompatible material (carrier); the use of animal models (syngenic, transgenic or cloned/transgenic suitable for xenotransplantation) as

temporary recipients as described; the use of segregated human tissue compartments as described.

According to a further aspect of the invention, there is provided a method for treating an animal or human by tissue engineering comprising surgically administering to a patient to be treated a cell/tissue/organ according to the invention.

Some examples of the therapeutic applications and value of this approach are the establishment, through the above technology, of autologous dopaminergic neurons or pre-neurons to be used for neurosurgical transplantation into the degenerated substantia nigra of patients affected by Parkinson's disease. Parkinson's disease is the result of progressive degeneration of dopaminergic neurons within the putamen area of the CNS. It has been already shown that neurons obtained from foetal brains, when surgically implanted, can restore the dopaminergic circuitry and have substantial long term clinical benefit in very advanced Parkinsonians. The strong limits of this therapeutic intervention are either the source of neurons and the immunological barrier (allogeneic neurons). This invention overcomes those limits entirely. Somatic quiescent cells (e.g. skin fibroblasts) or non-quiescent cells (e.g. haematopoietic stem cells such as CD34+ cells) can be easily and safely taken from a given Parkinson's patient, and they constitute the source of the nucleus. Once the nucleus is transferred into an enucleated oocyte (nuclear transfer technology), it reaches the morula stage which is then implanted subcutaneously into the patient (other alternative methods of protected implantation or the use of surrogate hosts are described above) and allowed to growth for a limited period (few weeks) up to the formation of precursor cells of any cell lineage. After its excision, multipotential neural stem cells can be isolated (ectoderm-like phenotype) from other cells precursors (mesoderm and endoderm lineages) by means of surface glycoprotein pattern, and further separated into neuronal-restricted precursor cells and glial-restricted precursor cells. These neuronal stem cells are then log-expanded in vitro using growth/differentiating factors (nerve growth factor-NGF, glial-derived neuronal factor -GDNF, retinoids and chemical neurotransmitters such as dopamine), tested

for microbial contamination and selected in appropriate number and function (dopamine production) for transplantation into the diseased CNS area (Substantia Nigra).

Other neurodegenerative disorders (Multiple Sclerosis, Amiothrophic Lateral Sclerosis) can be targeted in a similar way providing that the dopaminergic circuitry is complemented with serotoninergic, colinergic and GABA-ergic populations of neurons (neuronal-restricted precursor cells) in association with glial-restricted precursor cells.

The same applies for the transplantation of autologous neuronal-restricted and glial-restricted precursor cells to be used in patients with spinal cord injury for spinal cord repair and the replacement of CNS neurons and glial cells lost following ischemic and hemorrhagic stroke.

Another area of critical intervention is the establishment of autologous Langerhans beta-cells producing insulin or entire islets of Langerhans producing counteracting hormones such as glucagon, and their implantation in patients with severe and uncontrolled diabetes.

Yet another area of intervention is the establishment of striated myocardial cells to be injected, through coronary catheterism, into an acute area of myocardial infarction.

2. HSC as donor

According to this embodiment of the present invention there is provided a method for forming a pluripotent cell, the method comprising transferring a hematopoietic stem cell nucleus into a recipient oocyte and, before or after transfer, removing the oocyte nucleus.

In one embodiment of the invention, the donor cell and recipient oocyte are of human origin. In an alternative embodiment, the donor cell and recipient oocyte are of non-human mammalian origin.

Preferably the donor cell is cord blood haematopoietic stem cell.

Preferably the recipient oocyte is a metaphase II oocyte, an unactivated oocyte or a preactivated oocyte.

Preferably the recipient oocyte is enucleated prior to transfer of the non-quiescent stem cell nucleus. In an alternative embodiment of the invention, the nucleus of the recipient oocyte is removed subsequent to transfer of the non-quiescent stem cell nucleus.

According to another embodiment of the invention there is provided a pluripotent cell comprising the nucleus of a hematopoietic stem cell and the cytoplasm of an enucleated oocyte.

In one embodiment, a cell of the invention is used for the manufacture of a cell mass comprising ectodermal, mesodermal or endodermal cells for use in implant/transplant therapy.

Development of a cell according to the invention provides for the formation of a cell mass. Particularly it provides for the formation of a cell mass from which a blastula-type mass comprising ectodermal, mesodermal and/or endodermal cells may be derived for purposes further described below.

According to an alternative embodiment of the invention there is provided a pluripotent cell or a cell-line derived from any of the ectodermal, mesodermal and/or endodermal cells of a blastula-type mass formed by the method.

Preferably the pluripotential characteristic includes the ability to proliferate in culture in an undifferentiated state.

In one embodiment of the invention the cell or cell-line has the capacity to proliferate in continuous culture in an undifferentiated state for at least 6 months and ideally 12 months.

Preferably the pluripotent characteristic includes the expression of at least one selected marker of pluripotent cells, for example a cell surface marker selected from the group comprising: SSEA-1 (1), SSEA-3 (+), SSEA-4 (+), TRA-1-60 (+), TRA-1-81 (+), alkaline phosphatase (+) and combinations thereof.

According to an alternative embodiment of the invention, there is provided a method for preparing a cell or cell-line comprising dissociating the cell mass formed by the above method to obtain dissociated cells. Usually the method further comprises growing the dissociated cells in culture under conditions conducive to proliferation of the cells and optionally storing the cells.

According to another embodiment of the invention, there is provided a method for inducing differentiation of at least one pluripotent cell comprising culturing the cell under conditions conducive to the differentiation of the cell into at least one tissue (e.g. neural-committed stem cells or glial-committed stem cells) and optionally storing the differentiated tissue.

According to yet another embodiment of the invention, there is provided a tissue or organ derived from a cell or cell-line of the invention selected form neuronal, muscle (smooth, striated and/or cardiac), bone, cartilage, liver, kidney, respiratory epithelium, spleen, skin, stomach, intestine and epithelial tissue. Preferably, the product comprising a suspension of committed cells, tissue or organ is combined with a suitable excipient, diluent or carrier and provided for use in tissue transplantation.

In one embodiment of the invention, ectodermal, mesodermal and/or endodermal cells derived from a blastula-type mass of the invention and/or a pluripotent cell or cell-line derived from any of the ectodermal, mesodermal and/or endodermal cells of the blastula-type mass, are used for implant/transplant therapy by their direct introduction into the patient to be treated. In this embodiment, late-stage differentiation of the cell or cells into the required tissue type occurs *in-vivo* by orthotopic implant (i.e. ectodermal-derived stem cells into CNS/PNS).

According to a separate embodiment of the invention, there is provided a method to treat conditions or diseases requiring tissue and/or organ transplantation comprising providing at least one tissue type or organ according to the invention, optionally including a suitable excipient, diluent or carrier, introducing the tissue or organ into a patient to be treated and treating the patient under conditions which are conducive to the acceptance of transplanted tissue by the patient.

In one embodiment of the invention, the ectodermal, mesodermal, endodermal cell, cell-line, tissue or organ is autologous to the patient requiring treatment. Most preferably, the hematopoietic stem cell or the stem cell nucleus is obtained at birth of the subject and cryopreserved prior to use in the method.

In an alternative embodiment, the ectodermal, mesodermal, endodermal cell, cell-line, tissue or organ is allogeneic to the patient requiring treatment. In one embodiment the hematopoietic stem cell or stem cell nucleus is cryopreserved prior to use in the method.

Contrary to GB 2318578 which teaches that the donor cell from which the nucleus is derived must be in the quiescent state, the present invention does not impose this pre-requisite upon selection of the donor cell and exploits the biological advantages of HSC nuclei for use in nuclear transfer. Advantageously, HSC nuclei maintain a more naïve and plastic undifferentiated state and express the highest level of telomerase

activity, together with DNA hypomethylation pattern. They thus maintain the developmental potential and indefinite proliferative capacity required to form adequate derivatives of all three embryonic germ layers and subsequent tissue-committed stem cells also in a proper quantity. Most kinds of cell therapy reconstitution in regenerative medicine usually require a proper amount of cells. In the example of neuronal-committed stem cells for treating Parkinson's disease, the amount of cells to be neurosurgically implanted through guided needle injections, might be in the magnitude of 10^5 to 10^8/site.

Nuclear transfer of neonatal hematopoietic stem cell (HSC) nucleus into an enucleated donor oocyte or into an oocyte from which the oocyte nucleus is subsequently removed, and its *in-vitro* growth up to the Inner Cell Mass (ICM) and blastocyst stage provides the basis for the establishment of pluripotent cell-lines which, upon exposure to growth/differentiation factors, can lead to the production of selected differentiated tissue for use, *inter alia*, in transplantation therapy. An example of growth/differentiating agents to be used for producing neuronal-committed stem cells are IGF-1, NGF, GDNF, retinoids and neurotransmitters (such as dopamine, serotonine, glutamate and others in various combinations).

An alternative embodiment of the invention involves the direct use of ectodermal, mesodermal and/or endodermal cells derived from a cell mass or a pluripotent cell or cell-line derived from any of the ectodermal, mesodermal and/or endodermal cells of the cell mass in implant/transplant therapy. In this embodiment, differentiation of the cell or cells into the required tissue type occurs *in-vivo*.

The method of the present invention allows for post-natal orthotopic (i.e. neural-committed stem cells injected into diseased CNS/PNS areas, or endocrino-committed stem cells injected into diseased endocrine organs) autologous (self) reconstitution or allogeneic cell/tissue transplant (donor to recipient) to achieve in vivo anatomo-functional reconstitution. In one embodiment of the invention, the human pluripotent cells and their tissue-committed derivatives can be used for post-natal autologous

(self) reconstitution; the nucleus which is used for the nuclear transfer process is derived from a cryopreserved set of HSC of the same individual requiring the therapeutic intervention. Accordingly, no immune rejection is present and thus no immune suppression is required for a long lasting engraftment (the oocyte antigenic counterpart is diluted throughout divisions and will represent a minor antigenic pattern within the more differentiated cells).

It is well known that allogeneic material, if transplanted into another individual, may illicit a severe immune reaction in the host and thus be destroyed unless adequate, though toxic, immunosuppressive regimens are employed. The method of the present invention provides the important advantage that, due to developmental immaturity and higher plasticity of the HSC, the material for use in transplant is less immunogenic and with greater proliferative nature. This provides for a greater rate of success with allogeneic cell/tissue transplant for which a certain critical number of committed stem cells might be warranted to obtain a successful tissue reconstitution. The allogeneic route may be preferred in circumstances where an individual has a genetic predisposition to disease and autologous (self) material cannot be used.

It will be apparent to one skilled in the art that the method of the present invention holds great potential for use in transplantation medicine, regenerative medicine, drug discovery and development and the study of human developmental biology.

It will be apparent that the methods described above for collection of neonatal blood and selection of HSC, cryopreservation, enucleation of cells to yield 'karyoplasts and cytoplasts' and transfer of a donor nucleus to an oocyte similarly apply.

In this embodiment a blastula-type mass obtained as above is dissociated to obtain dissociated cells which are grown in suitable culture conditions. In one embodiment of the invention the culture conditions are conducive to proliferation and expansion of the cells. The cell culture may optionally be stored under suitable storage conditions.

In one embodiment, ectodermal, mesodermal and/or endodermal cells derived from a cell mass or a pluripotent cell or cell-line derived from any of the ectodermal, mesodermal and/or endodermal cells of the cell mass are used for implant/transplant therapy. Differentiation of the cell or cells into the required tissue type occurs *in-vivo*, providing that the appropriate layer cells are introduced into the right anatomical site (i.e. ectoderm cells or ectoderm-derived cells into CNS/PNS), thus avoiding the formation of a teratoma substrate.

Differentiation of at least one pluripotent cell of the invention may be induced by culturing the cell under conditions conducive to the differentiation of the cell into at least one tissue. In one embodiment of the invention, the differentiated tissue may be stored prior to use under suitable storage conditions.

A tissue or organ derived from a cell or cell-line of the invention may include neuronal, muscle (smooth, striated and/or cardiac), bone, cartilage, liver, kidney, respiratory epithelium, haematopoietic cell, spleen, skin, stomach, intestine tissue and endocrine tissue.

A tissue or organ obtained by the method of the invention may be combined with a suitable excipient, diluent or carrier and provided for use in tissue transplantation. The tissue or organ is introduced into a patient to be treated under conditions which are conducive to the acceptance of the transplanted tissue by the patient. Due to the pluripotent nature of a cluster of cells isolated from any of the germ layers, the autologous or allogeneic transplant should be done orthotopically (i.e. in the same anatomo-functional tissue sharing the same germ layer origin during embryogenesis). This will allow a further in-vivo growth and differentiation due to the presence of known and unknown tissue-specific growth/differentiating substances. This will also avoid a "reprogramming" path of the implanted pluripotent cells toward unwanted tissue types.

CLAIMS

1. A method of culturing cells derived by growing a cell mass to form a teratoma-like cell mass, to grow and/or differentiate said cells.

2. A method of claim 1 wherein the teratoma-like cell mass was prepared by providing an activated oocyte obtained by nuclear transfer from a somatic quiescent or non-quiescent donor cell to a recipient oocyte and, before or after transfer, removing the oocyte nucleus, providing conditions which are suitable for the division of the oocyte to at least a sixteen-cell mass stage, transplanting the cells into a recipient, wherein the cells are allowed to develop into a localised teratoma-like cell mass, the method further comprising excising the teratoma-like cell mass and putting it into *in-vitro* culture.

3. A method according to claim 1 comprising

(i) transferring the nucleus of a somatic donor cell into a recipient enucleated oocyte;

(ii) activating the oocyte;

(iii) providing conditions which are suitable for the division of the oocyte to at least a sixteen-cell mass (morula-like stage);

(iv) providing conditions which are suitable the sixteen-cell mass to develop into a localised teratoma-like cell mass;

(v) separating out differentiated cells of the cell mass, optionally either before or after culturing the cells in in-vitro conditions which are suitable for the further growth and/or differentiation thereof.

4. A method according to claim 1 or claim 2 comprising:

(i) obtaining a somatic cell, either quiescent or non-quiescent (e.g. haematopoietic stem cell from peripheral blood, bone marrow or from cryopreserved cord blood stem cells)

(ii) transferring the nucleus of the somatic cell into a recipient enucleated oocyte;

(iii) activating the oocyte;

(iv) providing conditions which are suitable for the division of the oocyte to at least a sixteen-cell mass (morula-like stage);

(v) transplanting the cells into a recipient (i) (i.e. subcutaneously);

(vi) allowing the cells in (v) to develop into a localised teratoma-like cell mass;

(vii) excising the teratoma – like cell mass in (vi); and

(viii) providing in-vitro conditions which are suitable for the further growth and/or differentiation of teratoma derived cells.

5. A method according to claim 3 or claim 4 wherein the somatic donor cell is a hematopoietic stem cell.

6. A method of producing a pluripotent cell comprising transferring a hematopoietic donor stem cell nucleus into a recipient oocyte and, before or after transfer, removing the oocyte nucleus.

7. A method according to any of claims 2 to 6 wherein the donor cell and recipient oocyte are of human origin.

8. A method according to any of claims 2 to 7 wherein the donor cell is a cord blood hematopoietic stem cell.

9. A method according to any of claims 2 to 8 wherein the recipient oocyte is a metaphase II oocyte, an unactivated oocyte or a preactivated oocyte.

10. A pluripotent cell derived by the method of any of claims 3 to claim 6 or any of claims 7 to 9 when dependent thereon.

11. A pluripotent cell comprising the nucleus of a hematopoietic stem cell and the cytoplasm of an enucleated oocyte.

12. Use of a cell according to claim 10 or claim 11 for the manufacture of a teratoma-like cell mass.

13. Use of a cell according to claim 11 for the manufacture of a cell mass comprising ectodermal, mesodermal or endodermal cells for use in implant/transplant therapy.

14. A teratoma-like cell mass derived from or composed of cells of claim 10 or claim 11.

15. A cell mass derived from or composed of cells of claim 10 or claim 11 comprising ectodermal, mesodermal and endodermal cells.

16. A cell or a cell-line derived from the teratoma-like cell mass of claim 14.

17. A cell or a cell-line derived from any of the ectodermal, mesodermal or endodermal cells of a cell mass of claim 15.

18. A cell or a cell-line of claim 16 or claim 17 wherein said cell or cell-line is a differentiated cell or cell-line and is selected from: neural cell; muscle cell (e.g. smooth, striated, cardiac); bone (osteoblast, osteoclast); chondrocyte; hepatocyte; renal; respiratory epithelium; haematopoietic cell; spleen cell; pancreatic islets (α, β cells); endothelial cell; cartilage; liver; kidney; respiratory epithelium; spleen; skin; stomach; intestine and epithelial.

19. A cell or cell-line according to any of claims 16 to 18 wherein the cell or cell-line has the capacity to proliferate in continuous culture in an undifferentiated state for at least 6 months and ideally 12 months.

20. A cell or cell-line according to any of claims 16 to 19 wherein the cell or cell-line is characterised by the expression of at least one selected marker of pluripotent cells which is a cell surface marker selected from the group comprising: SSEA-1 (1), SSEA-3 (+), SSEA-4 (+), TRA-1-60 (+), TRA-1-81 (+), alkaline phosphatase (+) and combinations thereof.

21. A method for preparing a cell or cell-line comprising;

(i) dissociating the cell mass of claim 14 or claim 15 to obtain dissociated cells; and optionally

(ii) growing the dissociated cells in culture under conditions conducive to proliferation of the cells; and optionally

(iii) storing the cell.

22. A method for inducing differentiation of at least one pluripotent cell of claim 10 or claim 11 or a cell or cell-line of any of claims 16 to 20, comprising;

(i) culturing the cell(s) under conditions conducive to the differentiation of the cell(s) into at least one tissue; and optionally

(ii) storing the differentiated tissue.

23. A tissue or organ derived from a cell of claim 10 or claim 11 or a cell or cell-line of any of claims 16 to 20 and selected from neuronal, muscle (smooth, striated and/or cardiac), bone, cartilage, liver, kidney, respiratory epithelium, spleen, skin, stomach, intestine and epithelial.

24. A product comprising a tissue or organ according to claim 23 and a suitable excipient, diluent or carrier.

25. A method to treat conditions or diseases requiring tissue and/or organ transplantation comprising introducing into a patient to be treated cells derived from a cell mass of claim 14 and/or 15 and/or a pluripotent cell or cell-line derived from any cells of the cell mass of claim 14 and/or 15, or a tissue or organ derived from a pluripotential cell of claim 10 and/or claim 11 or cell-line of any of claims 16 to 20, optionally including a suitable excipient, diluent or carrier.

26. A method of implant/transplant therapy comprising forming a pluripotent cell using a method of any of claims 1 to 9, forming a cell mass of claim 14 or claim 15 and performing a method comprising the method of claim 21 to obtain pluripotent cells or derivatives thereof for use in implant/transplant therapy.

27. A method of implant/transplant therapy comprising forming a pluripotent cell using a method of any of claims 1 to 9, forming a cell mass of claim 14 or 15 and performing a method comprising the method of claim 21 to obtain pluripotent cells, culturing the pluripotent cells under conditions conducive to the differentiation of the cells into at least one tissue or organ, and using the tissue or organ in implant/transplant therapy.

28. A method according to claim 27 wherein a hematopoietic stem cell is obtained at birth of the patient and cryopreserved prior to use of the cell in the method of the invention.

29. A method according to claim 28 wherein a hematopoietic stem cell nucleus is extracted from the patient and then cryopreserved prior to use of the nucleus in the method of the invention.

30. A method according to claim 29 wherein a pluripotent cell according to claim 10 or claim 11 is cryopreserved prior to use of the cell in the method of the invention.

31. A method according to any of claims 25 to 27 wherein the pluripotent cell, cell-line, tissue or organ is allogeneic to the patient requiring treatment.

32. A method according to claim 31 wherein the hematopoietic stem cell is cryopreserved prior to use of the cell in the method of the invention.

33. A method according to claim 31 wherein the hematopoietic stem cell nucleus is cryopreserved prior to use of the nucleus in the method of the invention.

34. A method according to claims 31 wherein a pluripotent cell according to claim 10 or claim 11 is cryopreserved prior to use of the cell in the method of the invention.

35. Use of a cell or cell-line of any of claims 16 to 20 in a method according to any of claims 25 to 34.

36. A method to obtain therapeutic polypeptides expressed by the cells according to the invention comprising:

iv) providing cell culture conditions suitable for the growth of a cell according to the invention;

v) isolating from said cell or the culture environment a therapeutic polypeptide; and, optionally;

vi) purifying and storing said therapeutic polypeptide.

37. A therapeutic composition comprising at least one therapeutic polypeptide according to the invention.

38. A method for treating an animal or human by tissue engineering comprising surgically administering to a patient to be treated a cell/tissue/organ according to the invention.

INTERNATIONAL SEARCH REPORT

International Application No
PC[illegible] 01/04229

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/06 A61K35/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HAIDI ZHANG ET AL: "Inhibition of tumorigenicity of the teratoma PC cell line by transfection with antisense cDNA for PC cell-derived growth factor (PCDGF, epithelin/granulin precursor)" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 95, November 1998 (1998-11), pages 14202-14207, XP002177206 ISSN: 0027-8424 the whole document --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 February 2002	15 February 2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nichogiannopoulou, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern··'----' Application No
PCT, .. 01/04229

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHOU J ET AL: "FIBROBLAST GROWTH FACTOR INHIBITS PROLIFERATION OF A HIGHLY TUMORIGENIC INSULIN-INDEPENDENT TERATOMA-DERIVED CELL LINE" GROWTH FACTORS, HARWOOD ACADEMIC PUBLISHERS GMBH, XX, vol. 9, no. 2, 1993, pages 123-131, XP001021652 ISSN: 0897-7194 the whole document	1
X	TOPP W ET AL: "IN VITRO DIFFERENTIATION OF TERATOMAS AND THE DISTRIBUTION OF CREATINE PHOSPHOKINASE AND PLASMINOGEN ACTIVATOR IN TERATOCARCINOMA-DERIVED CELLS" CANCER RESEARCH, AMERICAN ASSOCIATION FOR CANCER RESEARCH, BALTIMORE, MD, US, vol. 36, November 1976 (1976-11), pages 4217-4222, XP002939898 ISSN: 0008-5472 the whole document	1
P,X	WO 01 59076 A (VORTMEYER A OLDFIELD E (US); GOVERNMENT OF THE UNITE) 16 August 2001 (2001-08-16) the whole document	1,26-34, 36-38
A	STEVENS L C: "TESTICULAR, OVARIAN, AND EMBRYO-DERIVED TERATOMAS" CANCER SURVEYS, OXFORD, GB, vol. 2, no. 1, 1983, pages 75-91, XP001020771 the whole document	1

INTERNATIONAL SEARCH REPORT

In onal application No.
PCT/GB 01/04229

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 25-35 and 38 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1 completely, 26-34, 36-38 partially

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1 completely and 26-34, 36-38 all partially

   A method of culturing cells derived by growing a cell mass to form a teratoma-like cell mass, to grow and/or differentiate said cells. Therapeutic treatments based on these cells.

2. Claims: 2-5, 12, 14-24 all completely and 10, 25-38 all partially

   A method of culturing cells derived by growing a cell mass to form a teratoma-like cell mass, to grow and/or differentiate said cells, wherein the teratoma-like cell mass was obtained through nuclear transfer from a somatic cell to a recipient oocyte. Pluripotent cells and teratoma-like cell masses derived therefrom and therapeutic applications based thereon.

3. Claims: 6-9, 11, 13 all completely and 10, 25-38 all partially

   A method of producing a pluripotent cell comprising transferring a hematopoietic donor stem cell nucleus into a recipient oocyte, pluripotent cells derived therefrom and their uses.

INTERNATIONAL SEARCH REPORT

Internat[illegible] Application No
PCT 01/04229

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0159076 A	16-08-2001	AU 3691901 A	20-08-2001
		WO 0159076 A2	16-08-2001